REPORT

Role of nitric oxide in the regulation of T cell functions

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There is a close relation between T helper (Th) 1 cells and nitric oxide in disease. Thus it is possible that a reciprocal regulatory mechanism exists between them. This paper briefly describes the experimental studies which have helped elucidate the mechanism by which nitric oxide selectively enhances Th 1 cell proliferation and the potential effect of nitric oxide on regulatory T (Treg) cells. On the basis of the results the authors propose that nitric oxide represents an additional signal for the induction of T cell subset response, contributing to the increasingly complex network of immune regulation essential for health and disease.

Nitric oxide is associated with some of the most important immunopathologies, including rheumatoid arthritis, diabetes, systemic lupus erythematosus, and septic shock.[1–6] Conversely, nitric oxide is also a key effector molecule for the defence against intracellular pathogens including virus, bacteria, and parasites.[7–9] A common feature of these diseases is the prominent role of type 1 (both CD4+ and CD8+) T cells.[10–13] Type 1 T cells, exemplified by CD4+ helper T cells 1 (Th 1), characteristically produce interferon γ (IFNγ) which can strongly activate macrophages to produce high concentrations of nitric oxide via inducible nitric oxide synthase (iNOS). In contrast, Th 2 cells produce interleukin (IL)-4 and IL-5, which can inhibit the iNOS induction by IFNγ.[14 15] Th 1 cells are associated with inflammatory diseases and elimination of intracellular pathogens, whereas Th 2 cells are closely involved in allergy and expulsion of extracellular parasites.[16] This dichotomy of Th 1 and Th 2 cells is crucial to the balance of immune response and forms the basis of the current concept of immune therapy. Both type 1 and type 2 cells are derived from the same precursor and are differentiated into the two distinct lineages, principally under the influence of cytokines in the micro-environment. IL-12 drives the differentiation of type 1 cells during specific antigenic activation of precursor T cells (Tp), whereas IL-4 is the main driving cytokine for the differentiation of type 2 cells.[17 18] Given the close relation between Th 1 cells and nitric oxide in disease, it is likely that there exists a reciprocal regulatory mechanism between them.

We have previously shown[19] that nitric oxide had a selective enhancing effect on the induction and differentiation of Th 1 but not Th 2 cells. The effect of nitric oxide was by acting directly on T cells, but in synergy with IL-12 produced by antigen presenting cells. Here we describe the mechanism by which nitric oxide selectively enhances Th 1 cell proliferation and the potential effect of nitric oxide on regulatory T (Treg) cells.

NITRIC OXIDE ENHANCES DIFFERENTIATION OF TYPE 1 T CELLS

Previously, we have demonstrated that although high doses of nitric oxide were cytotoxic, low doses of nitric oxide selectively enhanced the differentiation of murine Th 1 but not Th 2 cells.[19] We have since investigated if this phenomenon is also applicable to subsets of CD8+ cells (Tc 1 and Tc 2) and human CD4+ T cells. Purified BALB/c CD8+ cells were cultured with immobilised anti-CD3 antibody and anti-CD28 in the presence of IL-12 plus anti-IL-4 (Tc 1 cells) or IL-4 plus anti-IL-12 and anti-IFNγ (Tc 2 cells).[20 21] Graded concentrations of the nitric oxide donor, NOC-18, were added at the beginning of culture and cellular proliferation, and IFNγ and IL-5 production in the supernatant determined by enzyme-linked immunosorbent assay (ELISA) four days into the culture. At 10 μM, NOC-18 markedly enhanced the differentiation of Tc 1 cells but not Tc2 cells. This enhancing effect was absent at 100 μM NOC-18. The cellular proliferation was mirrored by the production of cytokines. Tc 1 cells differentiated in the presence of 10 μM of NOC-18 produced significantly more IFNγ compared with Tc 1 treated with 100 μM NOC-18 or medium alone. There was no evidence of increased IL-5 synthesis during Tc 2 differentiation at all the doses of NOC-18 tested. The increased IFNγ concentration in the culture supernatant is likely due to increased number of type 1 T cells rather than the larger amount of the cytokine produced by individual cells. Flow cytometric analysis of intracellular IFNγ revealed that there was no evidence of increased staining intensity of Th 1/Tc 1 cells in the cultures treated with 10 μM of NOC-18 compared with the medium control.

We then investigated whether this enhancing effect of low concentrations of nitric oxide also applied to human T cells. CD4+ T cells were purified from human cord blood and driven to the Th 1 cell lineage by culturing with phytohaemagglutinin in the presence of human IL-12 and anti-IL-4 as described previously.[22 23] Graded concentrations of NOC-18 were added at the beginning of the culture, which was terminated on day 5. The differentiation of human Th 1 cells was markedly enhanced by the presence of 5 μM NOC-18 and the development was clearly suppressed by higher concentrations (>40 μM) of NOC-18 compared with medium control. These data therefore extend our earlier finding on the selective enhancing effect of low doses of nitric oxide on murine Th 1 cells to Tc 1 cells, and also to human T cells. Furthermore, it appears that human CD4+ T cells are even more sensitive to the varying concentrations of nitric oxide than murine T cells.

EFFECT OF NITRIC OXIDE ON TYPE 1 T CELLS IS CGMP DEPENDENT

We next investigated the mechanism involved in the enhancing effect of low concentrations of nitric oxide on type 1 cell differentiation. Since nitric oxide is known to influence apoptosis, the most straightforward explanation for

Abbreviations: Ag, antigen; cGMP, 3′,5′-cyclic guanosine monophosphate; IL-12R, interleukin-12 receptor; iNOS, inducible nitric oxide synthase; NOC-18, 2,2′-(hydroxynitrosohydrazino)bis-ethamine; ODQ, 1-H-oxodiazolo-(1,2,4)-(4,3-a) guinoxaline-1-one; sGC, soluble guanylyl cyclase; TCR, T cell receptor; Th, helper T cell; Tc, cytotoxic T cell; Tp, T cells precursor; Treg, regulatory T cell

our finding would be that low doses of nitric oxide inhibit T cell apoptosis and thereby contribute to the increased viability of the treated cells. In repeated experiments, there was no evidence that low doses of nitric oxide (5–25 μM NOC-18) significantly influenced the apoptosis of T cells, although at higher concentrations (>100 μM, NOC-18) nitric oxide consistently increased the level of apoptosis. We then examined the possibility that nitric oxide may exert its influence via the activation of sGC (soluble guanylyl cyclase) thus elevating cGMP (3′,5′-cyclic guanosine monophosphate), a well established nitric oxide effector pathway. We measured the levels of intracellular cGMP in CD4+ T cells cultured for 30 minutes under the Th 1 driving condition in the presence of graded concentrations of NOC-18. cGMP concentration was significantly elevated by 5 μM and 10 μM of NOC-18. This amount declined to the control level at 100 μM of NOC-18. The pattern of cGMP elevation closely correlated with the enhanced Th 1 cell activation by nitric oxide. We then tested the effect of ODQ [(1-H-oxodiazolo-(1,2,4)-(4,3-a) guinoxaline-1-one)], a competitive inhibitor of the activation of cGMP. ODQ oxidises the ferrous iron of sGC to ferric iron and so prevents the effective binding of nitric oxide to sGC, and is thus a unique and potent inhibitor of the nitric oxide/cGMP pathway. CD4+ T cells were purified from D011.10 OVA-TCRαβ transgenic mice (BALB/c background) and cultured with the ovalbumin (OVA) peptide and mitomycin C treated BALB/c spleen cells (antigen presenting cells) for four days in the presence of IL-12 plus anti-IL-4 antibody (Th 1), or IL-4 plus anti-IL-12 and anti-IFNγ antibodies (Th 2) and graded concentrations of NOC-18. ODQ (10–40 μM) was added at the beginning of the culture. ODQ abrogated the enhanced Th 1 differentiation induced by 20 μM NOC-18. In contrast, ODQ had no effect on the induction of Th 2 cells across a range of NOC-18 concentrations. In additional experiments, polyclonally activated (anti-CD3 stimulated) Th 1 cells were also investigated. The enhanced Th 1 cell development in the presence of 20 μM NOC-18 and in the absence of antigen presenting cells was also completely blocked by ODQ. These results therefore strongly indicate that the enhancing effect of low concentrations of nitric oxide is mediated by cGMP. Furthermore, this effect is exerted directly and selectively on Th 1 cells and not via antigen presenting cells.

NITRIC OXIDE SELECTIVELY ENHANCES THE EXPRESSION OF IL-12Rβ2

We next investigated the mechanism by which cGMP selectively enhances Th 1 cells differentiation. As nitric oxide only increased Th 1 cell differentiation and had no effect on established Th 1 clones or lines, and as it acted on the T cell directly and in synergism with IL-12,[19] we reasoned that nitric oxide might enhance expression of the inducible IL-12Rβ2 via cGMP. To test this possibility, CD4+ or CD8+ T cells from BALB/c mice were driven to polyclonal Th 1/Tc 1 or Th 2/Tc 2 lineages using immobilised anti-CD3 antibodies in the presence of the appropriate cytokines. NOC-18 (10 μM) was added at the beginning of the cultures, which were harvested at 16 hours. mRNA was extracted and the levels of IL-12Rβ2, IL-18Rα, and IL-4R were quantified by real time polymerase chain reaction (PCR) (Taqman). Th 1 and Tc 1 cells treated with NOC-18 showed significantly higher levels of IL-12Rβ2 message compared with those not treated with nitric oxide. In contrast, the levels of IL-4R message in the Th 2/Tc 2 cells were not affected by the treatment with nitric oxide. Interestingly, the levels of IL-18Rα, characteristic of Th 1/Tc 1 cells,[24] were also not affected by the treatment with low doses of nitric oxide. These data therefore strongly indicate that nitric oxide selectively enhanced the expression of IL-12Rβ2 during type 1 cell differentiation, and that this may account for the preferential induction of type 1 cell development by low dose of nitric oxide.

NITRIC OXIDE INDUCES A POPULATION OF Treg CELLS

Th 1 cells are key players in the host immune defence against pathogens as well as causing a range of autoimmune inflammatory conditions, some of which are dependent on nitric oxide. We wondered how this nitric oxide–Th 1 self-amplification cycle might be regulated. A potential candidate would be the Treg cell. We therefore investigated the effect of nitric oxide on CD4+CD25+ Treg cells.

There is considerable current interest in the functional role of regulatory T cells, which subsume the role, if not the characteristics, of the much-maligned suppressor T cells. There are at least three major types of Treg cell: Th 3, Tr 1 and CD4+CD25+ T cells with overlapping functions.[25–27] CD4+CD25+ Treg cells are arguably the best characterised, principally because it is relatively easy to obtain large number of cells. The main characteristic of Treg cells is the expression of the intracellular X-linked forkhead/winged helix transcription factor, Foxp3.[28–30]

CD4+CD25+ and CD4+CD25− T cells were purified from the lymph nodes of BALB/c mice and cultured with soluble anti-CD3 (sαCD3) antibody and spleen cells treated with mitomycin-C (as antigen presenting cells) [a culture condition known to be optimal for the suppressive function of Treg cells[31]] in the presence of NOC-18. Nitric oxide had little or no effect on the proliferation of the CD4+CD25+ T cells. In contrast, nitric oxide markedly increased the proliferation, division and viability of CD4+CD25− T cells. There was a transient delay of cell proliferation on day 2. Thereafter, the cells continued to divide reaching 40× the original number by day 6 and remained up to 95% viable by days 10–14 of culture. The increase in cell proliferation was effective only when nitric oxide was added soon (<6 hours) after the activation of the T cell receptor (TCR) but was independent of cGMP. Nitric oxide alone was without effect. These results therefore reveal a previously unrecognised function of nitric oxide, which in conjunction with TcR triggering, selectively enhances the differentiation and survival of a population of CD4+CD25− and CD8+ T cells. Impressively, the nitric oxide expanded CD4+CD25− T cells are almost 100% CD25+.

The conversion of CD4+CD25$^-$ cells to CD4+CD25+ T cells by NO prompted us to investigate the suppressive function of this population of cells with the Treg phenotype. Freshly purified CD4+CD25− cells (responders) were co-cultured with nitric oxide induced CD4+CD25+ T (NO-Treg) cells or freshly purified CD4+CD25+ T (natural Treg) cells in the presence of sαCD3 and antigen presenting cells. NO-Treg cells were as efficient as the natural Treg cells in suppressing the differentiation of the responders.

DISCUSSION

A strong immune regulatory role for nitric oxide has long been recognised.[32 33] Earlier studies showed that mice deficient in nitric oxide developed enhanced Th 1 response following infections and antigenic stimulation, producing more IFNγ and less IL-4 compared with intact mice treated similarly.[34–36] These observations indicated that nitric oxide selectively inhibits the expansion of Th 1 cells by a negative feedback mechanism. This is achieved at least in part by the selective inhibition of IL-12 synthesis by activated macrophages.[37] This mechanism would potentially be highly beneficial during inflammatory diseases predominantly mediated by type 1 cells. In contrast, a strong type 1 T cell response is highly desirable for an effective host defence against intracellular pathogens. This could be enhanced and accelerated by low concentrations of nitric oxide provoked

during the early stage of infection.[19] Thus, immunologically, nitric oxide may have evolved to fine tune the immune homoeostasis in health and disease. Low doses of nitric oxide preferentially promoted type 1 T cell differentiation by selective induction of IL-12Rβ2 via cGMP.

Among the several factors reported to be important for inducing type 1 T cell differentiation,[38–40] IL-12 clearly plays the dominant role.[17 18] IL-12R is composed of two subunits, β1 and β2 which are both required for high affinity IL-12 binding and signalling.[39–41] IL-12Rβ1 is widely expressed in cells of haematopoietic origin, whereas IL-12Rβ2 is detected selectively in cells responsive to IL-12 such as T cells. Naive T cells express IL-12R on TCR engagement. The expression of IL-12R appears to be transient and is downregulated in committed memory Th 1 cells,[41] which can be maintained and expanded by other factors including IL-15.[42–44] However, the initial expression of IL-12R is sustained by the presence of the innately induced cytokine, IL-18.[24] This has been used to explain the often-observed synergistic effect of IL-18 and IL-12 in the induction of type 1 T cells.[45 46] Nitric oxide therefore represents an additional factor for enhancing IL-12R expression and thus promoting type 1 cell differentiation.

The mechanism by which cGMP selectively induces IL-12Rβ2, rather than IL-4R or IL-18R is at present unclear. In endothelial cells[47] and in macrophages,[48] low concentrations of nitric oxide were shown to upregulate and high doses of nitric oxide downregulate nuclear factor (NF)-κB activity. Alternatively, cGMP may act through the P-Raf/MEK/ERK pathway. The precise mechanism(s) of the action of low concentrations of nitric oxide in the type 1 T cell differentiation system is currently being explored.

The following molecular mechanism seems likely. Nitric oxide, produced by activated antigen presenting cells or provided exogenously in the microenvironment, activates sGC and leads to increased cGMP production. This then leads to the induction of increased IL-12Rβ2 expression, which facilitates the differentiation of type 1 T cells by IL-12 and TCR engagement. IL-12 is produced by the antigen presenting cells or present exogenously in the microenvironment. These findings therefore provide an explanation for the hitherto puzzling but important observation that low concentrations of nitric oxide enhance T cell proliferation and that this enhancement is selective for the induction of Th 1 cells, but not Th 2 cells, or established memory Th 1 cells. Nitric oxide therefore represents an additional signal for the induction of T cell subset response, contributing to the increasingly complex network of immune regulation essential for health and disease.

Excessive Th 1 amplification could lead to autoimmune diseases. To avoid this occurrence, nitric oxide also induces a population of Treg cells which could dampen the potentially damaging autoimmune response. Although the mechanism by which nitric oxide converts CD4+CD25− effector cells to a population of CD4+CD25+ Treg cells is currently unclear, the nitric oxide induced Treg cells may represent an important means by which the mammalian hosts regulate their immune response to achieve a state of homoeostasis via the key mediator, nitric oxide.

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